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Circadian Clock Neurons in the Silkmoth Antheraea pernyi: Novel Mechanisms of Period Protein Regulation

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Summary

We examined Period (PER) protein regulation in the brain of the silkmoth Antheraea pernyi. PER expression is restricted to the cytoplasm and axons of eight neurons, with no evidence of temporal movement into the nucleus. These neurons appear to be circadian clock cells, because PER and *per* mRNA are colocalized and their levels oscillate in these cells, Timeless protein immunoreactivity is coexpressed in each PER-positive neuron, and clock protein and mRNA oscillations are all suppressed in these neurons by constant light. A *per* antisense RNA oscillation was detected that is spatially restricted to PER-expressing cells, suggesting a novel mechanism of PER regulation. PER-positive neurons and their projections are strategically positioned for regulating prothoracicotropic hormone and eclosion hormone, two neurohormones under circadian control. Differences in the molecular details of PER expression and regulation between the brains of silkmoths and fruitflies provide insights into the mechanisms of clock gene regulation.

Introduction

The *period* (*per*) locus of Drosophila melanogaster is necessary for circadian rhythms in adult eclosion behavior and locomotor activity. Primary evidence for an essential role of *per* in circadian function came from mutation analysis in which nonsense mutations of *per* cause arrhythmicity (per^0), while missense mutations can either lengthen (per^L) or shorten (per^S) the period of circadian rhythms (Konopka and Benzer, 1971).

The leading hypothesis of how PER functions in circadian timekeeping is that PER acts as a negative regulator of its own transcription, forming an autoregulatory feedback loop that constitutes a molecular gear of a circadian clock (Hardin et al., 1990). Evidence in Drosophila supporting this hypothesis is substantial (reviewed by Hall, 1995; Kay and Millar, 1995; Reppert and Sauman, 1995). *per* mRNA and protein levels exhibit prominent daily rhythms with peak mRNA values preceding peak protein levels by several hours. PER is found in the nucleus, and its nuclear entry appears to be temporally controlled. Analysis of the entry of PER into the nuclei of Drosophila brain "lateral neurons" (the presumed site of circadian pacemaker cells; Frisch et al., 1994) shows that the protein first accumulates in the cytoplasm and then enters the nucleus during a restricted part of the circadian cycle (Curtin et al., 1995). Once in the nucleus, however, the mechanism by which PER alters *per* transcription remains a mystery.

A second clock gene, *timeless* (*tim*), was recently cloned from Drosophila (Gekakis et al., 1995; Myers et al., 1995). Importantly, TIM appears to regulate the time-dependent nuclear entry of PER (reviewed by Reppert and Sauman, 1995). The nuclear entry of TIM is also temporally gated, and TIM appears to function in its own autoregulatory feedback loop to repress transcription (Sehgal et al., 1995). Thus, molecular models of a Drosophila clock must now take into consideration the interdependent molecular loops of PER and TIM. The recent demonstration that TIM levels respond to light in a time-dependent manner suggests that the manipulation of PER–TIM dimers by photic stimuli may account for clock resetting effects of light in Drosophila (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996).

An issue of central importance is whether Drosophila circadian clock genes and their molecular mechanisms are evolutionarily conserved. Work in this area has led to the cloning of *per* from other dipterans (Colot et al., 1988; Nielsen et al., 1994) and insects outside the order Diptera (Reppert et al., 1994), extending the generality of the potential clock function of PER. Important in this regard, *per* has been cloned from the giant silkmoth Antheraea pernyi, an insect that diverged from dipterans 240 million years ago (Reppert et al., 1994). The silkmoth *per* cDNA encodes a protein that shares sequence identity with fly PER in several regions. *per* mRNA levels exhibit a prominent circadian oscillation in silkmoth heads, and PER levels manifest a robust daily variation in silkmoth photoreceptor nuclei. Expression of the silkmoth *per* cDNA in per^0 transgenic flies showed that the silkmoth homolog can function as a circadian clock element in Drosophila (Levine et al., 1995).

With the cloning of *per*, A. pernyi becomes an interesting alternative to Drosophila for the study of clock gene mechanisms. Silkmoths are holometabolous insects, like fruit flies, and they manifest robust circadian behaviors (Truman and Riddiford, 1970; Truman, 1972, 1974). Moreover, elegant brain lesion and transplantation studies in A. pernyi in the early 1970s showed that a circadian clock controlling the timing of the photoperiodic termination of pupal diapause, adult eclosion, and the adult flight rhythm resides in the dorsal lateral protocerebrum (Truman and Riddiford, 1970; Truman, 1972, 1974; Williams and Adkisson, 1964). The larger brain of the silkmoth also allows a level of study of putative circadian clock cells, such as electrophysiological analysis, not readily achieved in Drosophila. Finally, silkmoths offer substantial potential for understanding the coupling between a circadian clock and output pathways, because several of the neuroendocrine factors under circadian control (e.g., eclosion hormone [EH] and prothoracicotropic hormone [PTTH]) have been characterized and cloned in lepidoptera (Kawakami et al., 1990; Truman, 1992; Sauman and Reppert, 1996).

In the present report, we examine mechanisms of PER regulation in circadian clock cells in the brain of A. pernyi. We find dramatic differences in the molecular details

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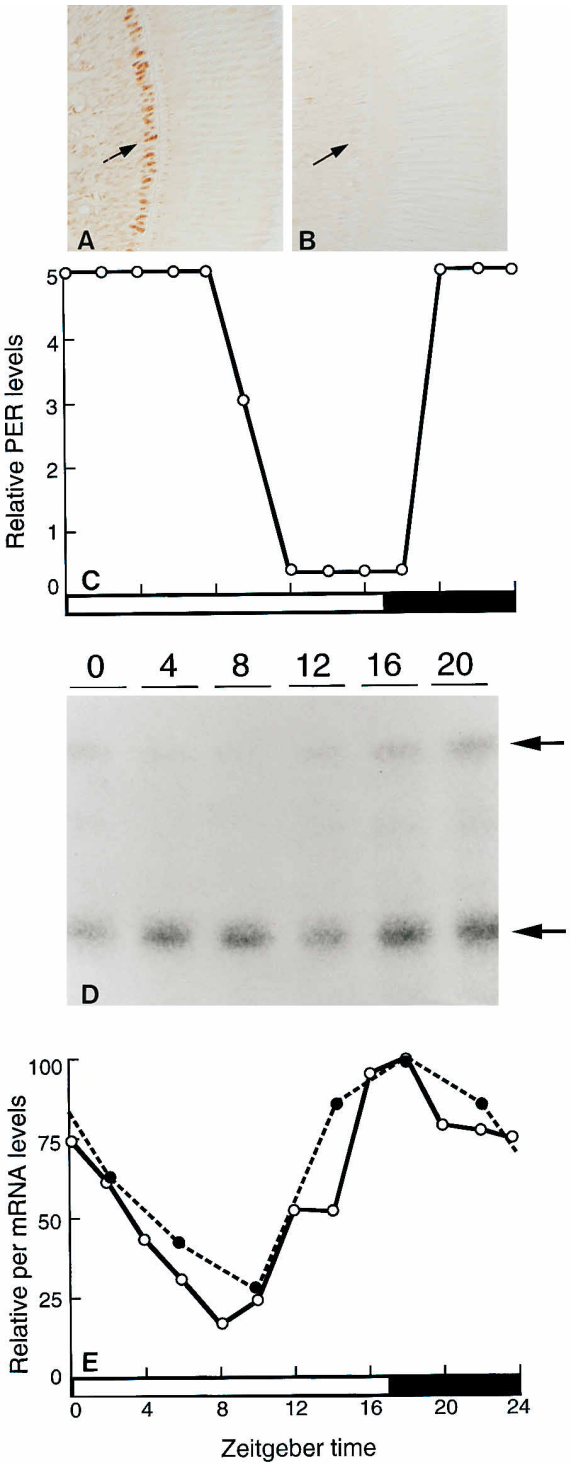

Figure 1. Cycling of PER and *per* mRNA Levels in A. pernyi Eyes

(A and B) Photomicrographs depicting the daily rhythm in PER staining in photoreceptor nuclei. (A), ZT 0; (B), ZT 14. Arrows depict nuclear layer. Magnification, 105×.

(C) Semiquantitative assessment of PER immunostaining in photoreceptor nuclei throughout LD 17:7. Each value is the mean of three animals. No value varied from the mean by more than one level of intensity at each time. A similar pattern was observed in two other experiments.

(D) RNase protection assay of *per* mRNA levels examined throughout the lighting cycle. ZTs are depicted above each lane. Upper arrow, *per* mRNA; lower arrow, RP49.

(E) Quantitation of RNase protection data for two separate experiments. Relative RNA levels refer to ratios of *per*:RP49 mRNAs that were converted to percentage of maximal level for each experiment. The horizontal bar at the bottom of (C) and (E) represents the LD 17:7 lighting cycle; open bars indicate light.

of PER expression and regulation between silkmoth and fruitfly brains. These differences provide important insights into the mechanisms of clock gene function.

Results

per mRNA and Protein Levels Oscillate with a Temporal Delay in Photoreceptor Nuclei

It was previously shown that *per* mRNA levels oscillate in the whole head (including brain and eyes) of A. pernyi and that PER-like immunoreactivity fluctuates (a two timepoint study) in silkmoth photoreceptor nuclei (Reppert et al., 1994). We have extended this study by performing a more detailed analysis of the temporal patterns of PER and *per* mRNA levels in silkmoth eye.

PER immunoreactivity in photoreceptor nuclei was examined over 24 hr at 2 hr intervals in light:dark (LD) 17:7 using an affinity-purified anti-PER antibody (58/10w) directed against the "peptide S" region of A. pernyi PER (for details, see Experimental Procedures). PER immunoreactivity in photoreceptors exhibited a robust daily rhythm (Figures 1A–1C). The rhythm was characterized by intense staining in photoreceptor nuclei from Zeitgeber time (ZT) 20 to ZT 8 (where ZT 0 equals lights-on). In contrast, no PER immunoreactivity was detected late in the light portion of the LD cycle (from ZT 12 to ZT 18).

per mRNA oscillations were examined over 24 hr at 2–4 hr intervals in LD 17:7. RNA from eyes (separated from brain) was examined by RNase protection analysis using a *per* cRNA probe from nucleotides 1009 to 1215 (Per-SmPAS; see Figure 2) of the silkmoth *per* cDNA. A *per* RNA fragment of appropriate size (207 nt) was protected in a rhythmic manner in silkmoth eyes by the antisense RNA probe (Figures 1C and 1D), with peak mRNA levels occurring at ZT 16 to ZT 18 and lowest levels from ZT 6 to ZT 10. The magnitude of the oscillation was 4- and 6-fold for the two separate experiments shown in Figure 1E. RNA for silkmoth ribosomal protein (RP49), which served as a control for the amount of RNA loaded in each lane, did not exhibit a daily rhythm in this or any other RNase protection experiments. When the temporal profiles of *per* mRNA and protein were compared, the increase in mRNA levels (by RNase protection assay) precedes the increase in protein levels in the nucleus (by immunocytochemistry) by 4–6 hr (compare Figures 1C and 1E).

PER Expression in Silkmoth Brain Oscillates and Is Limited to Eight Cells

In A. pernyi, the central brain contains a circadian clock that drives circadian rhythms in adult eclosion and adult flight (Truman and Riddiford, 1970; Truman, 1972, 1974). Thus, we used PER immunocytochemistry to identify

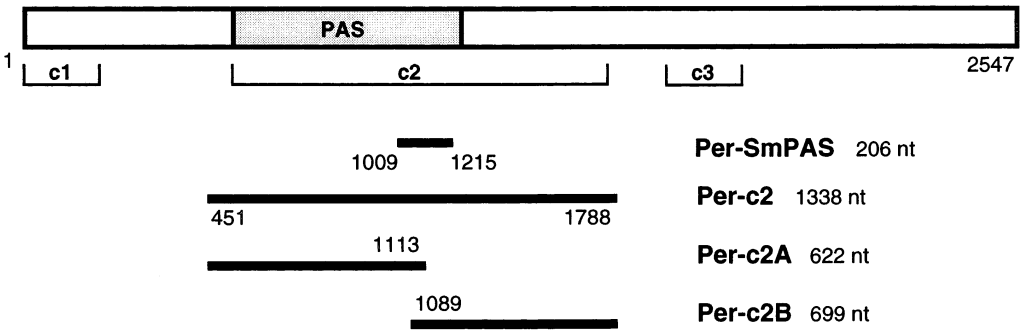

Figure 2. Location of RNA Probes Used for In Situ Hybridization and RNase Protection Assays

The A. pernyi *per* coding region is shown in the top portion of the figure with the PAS domain and conserved regions (c1–c3; see Colot et al., 1988; Reppert et al., 1994) highlighted. Solid horizontal lines depicts the location of each probe relative to the coding region. Nucleotide numbers are shown with 1 representing the first nucleotide of the coding region.

putative circadian clock cells in silkmoth brain. Six anti-PER antibodies (four against Drosophila PER and two against silkmoth PER) were examined for their ability to identify PER-expressing cells in silkmoth brain (for a list of the antibodies used, see Experimental Procedures). All anti-PER antibodies labeled four cells in each brain hemisphere (Figure 3), with one lateral pair and one medial pair in the dorsolateral region of each hemisphere (Figure 4A). Eight PER-positive cells were consistently found in A. pernyi brain throughout pupal and adult development (e.g., Figure 3 is pupal brain, and Figures 4–9 are adult brains). The number of PER staining cells did not vary over the course of the day, and there were no other PER-positive cells identified in silkmoth brain with any of the antibodies.

A striking feature of the PER-positive neurons in brain was intense staining in the cytoplasm and scant to undetectable staining in the nucleus. The intense cytoplasmic staining and lack of nuclear staining gave the stained cells a characteristic "doughnut" appearance (Figures 3 and 4). Examination of PER immunoreactivity in brain over 24 hr at 2 hr intervals in LD 17:7 revealed an oscillation of PER immunoreactivity in the cytoplasm of each cell (Figure 4D); there was no detectable oscillation of PER staining in the nucleus. The oscillation in cytoplasmic PER staining was characterized by peak levels between ZT 16 and ZT 22, with low levels between ZT 4 and ZT 8.

In addition to the fluctuation of PER staining in the cytoplasm, there was a pronounced oscillation of PER immunoreactivity in the axonal projections of the eight cells (compare Figures 4A and 4C; for time course, see Figure 4D). When PER immunoreactivity was highest in the cytoplasm, intense staining was also apparent in axons of PER-immunoreactive cells (Figure 4A). These axons could be frequently traced all the way to the ipsilateral corpora allata (Figure 4B). In addition, PER-positive axonal projections from the lateral and medial PER-positive cell pairs merged to form a single axonal tract on each side (Figure 4A). When PER staining was low in cytoplasm, there was no visible axonal staining (Figure 4C). Thus, the oscillation in PER staining in brain was most striking in axons.

The pattern and cellular localization of PER expression in A. pernyi brain is not unique to this silkmoth species, because similar results were observed in other saturniid moths. In Hyalophora cecropia, Actias luna, and Antheraea polyphemus, for example, PER expression was restricted to eight neurons in each silkmoth brain, in the same locations where PER-positive cells reside in A. pernyi. Moreover, PER staining in these other saturniid brains was also cytoplasmic and not nuclear, and there was axonal staining for PER similar to that described for A. pernyi.

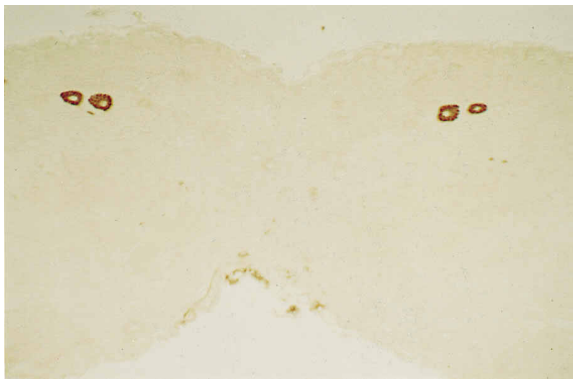

Figure 3. PER Immunoreactivity Is Restricted to Eight Cells in A. pernyi Brain

PER staining of a pair of lateral cells in each dorsolateral protocerebrum of pupal brain detected with the Young anti-PER antibody. The medial cells are not present in this plane of section. Similar results were obtained with five other anti-PER antibodies in pupal and adult brains. Magnification, 70×.

PER and *per* mRNA Are Colocalized in Brain

We next examined whether PER-containing cells in A. pernyi brain are also PER-producing cells and whether mismatches exist between protein and mRNA expression. In situ hybridization with a specific digoxigenin-labeled antisense cRNA probe (Per-c2; see Figure 2) corresponding to the c2 region of A. pernyi *per* revealed a hybridization signal restricted to eight neurons in each

silkmoth brain, in the same locations where PER-immunoreactive cells reside. No hybridization signal was detected in other cells throughout brain examined over the course of the LD cycle.

To examine whether the four pairs of neurons detected by immunolabeling are identical to the cells visualized by in situ hybridization, we performed double-labeling experiments with both the anti-PER antibody and the *per* cRNA probe. Eight cells (four in each hemisphere) and their axonal projections were clearly stained by immunofluorescence for PER. The same eight cells also expressed a specific hybridization signal detected with the digoxigenin *per* antisense cRNA probe (Figures 5A and 5B); the hybridization signal was limited to the cell body.

per mRNA Levels Oscillate in Brain

To examine the temporal pattern of *per* mRNA abundance in brain, we used RNase protection assay. Dissected brains were collected over 24 hr at either 2 or 4 hr intervals in LD 17:7. For each of three experiments, the silkmoth *per* RNA fragment from brains was protected by antisense RNA probe (Per-SmPAS) in a rhythmic manner (Figures 5C and 5D), with high *per* mRNA levels between ZT 14 and ZT 22 and low levels during the day (ZT 4 to ZT 10). The magnitude of the oscillation varied from 3- to 6-fold among the three separate experiments. When the *per* mRNA and protein rhythms in brain were compared (Figures 4D and 5D), they appear synchronous, with no apparent temporal delay between the two rhythms.

per Antisense RNA Levels Oscillate in Brain

When the in situ hybridization experiments of *per* expression in silkmoth brain described above were performed, sense cRNA probes were also used as a negative control. Surprisingly, the sense *per* RNA probe (Per-c2 fragment) gave an intense hybridization signal in the cytoplasm of the same eight cells that express PER; cellular coexpression of the sense and antisense transcripts was confirmed by double-labeling experiments with anti-PER antibody (Figures 6A–6D). There was also a striking oscillation in the hybridization signal from the sense probe, with the peak signal opposite the hybrization peak with the antisense probe (Figures 6A, 6B, and 6F). Specifically, the sense probe produced an intense signal from ZT 4 to ZT 8 and no detectable signal from ZT 16 to ZT 20. RNase protection studies using the sense cRNA probe (Per-c2) revealed a protected fragment of ~175 nt that was rhythmically protected throughout the LD 17:7 cycle (Figures 6E and 6F). The phases of the antisense RNA oscillations by in situ hybridization and RNase protection assay were very similar to each other and antiphase to the sense RNA oscillation in silkmoth brain (compare Figures 5D and 6F).

To provide further information as to the portion of the *per* gene giving rise to the antisense mRNA, we performed RNase protection assays with two shorter sense cRNA probes (Per-c2A and Per-c2B; see Figure 2) that spanned the length of the Per-c2 probe. A clear oscillating signal of ~175 bp and of appropriate phase

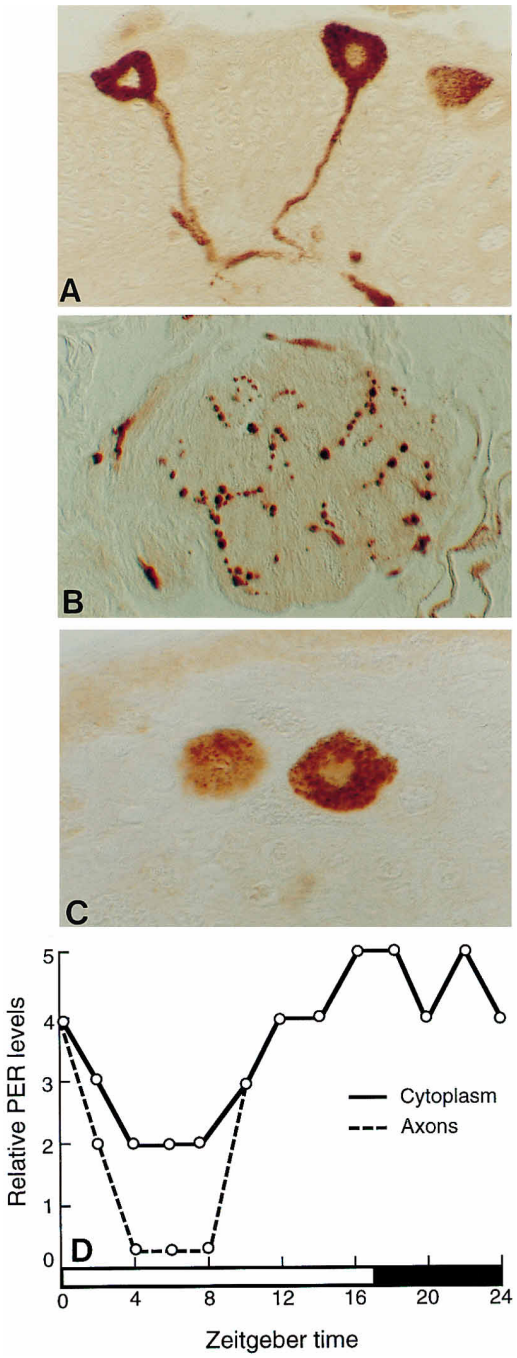

Figure 4. Daily Oscillation of PER Staining in Axonal Projections of PER-Immunoreactive Cells in A. pernyi Brain

(A) PER staining in coalescing axons from one cell from the lateral pair and one from the medial pair at ZT 22 in adult brain. Magnification, 345×.

(B) High magnification of corpus allatum showing PER immunoreactivity in axons at ZT 22. Magnification, 670×.

(C) PER staining is limited to the cell body at ZT 6. Axonal staining was not detected for PER in any of the series of adjacent sections. Magnification, 370×.

(D) Semiquantitative assessment of PER immunostaining in brain cells throughout LD 17:7. Each value is the mean of three animals. No value varied from the mean by more than one level of intensity at each time. A similar pattern of immunoreactivity was observed in two other experiments.

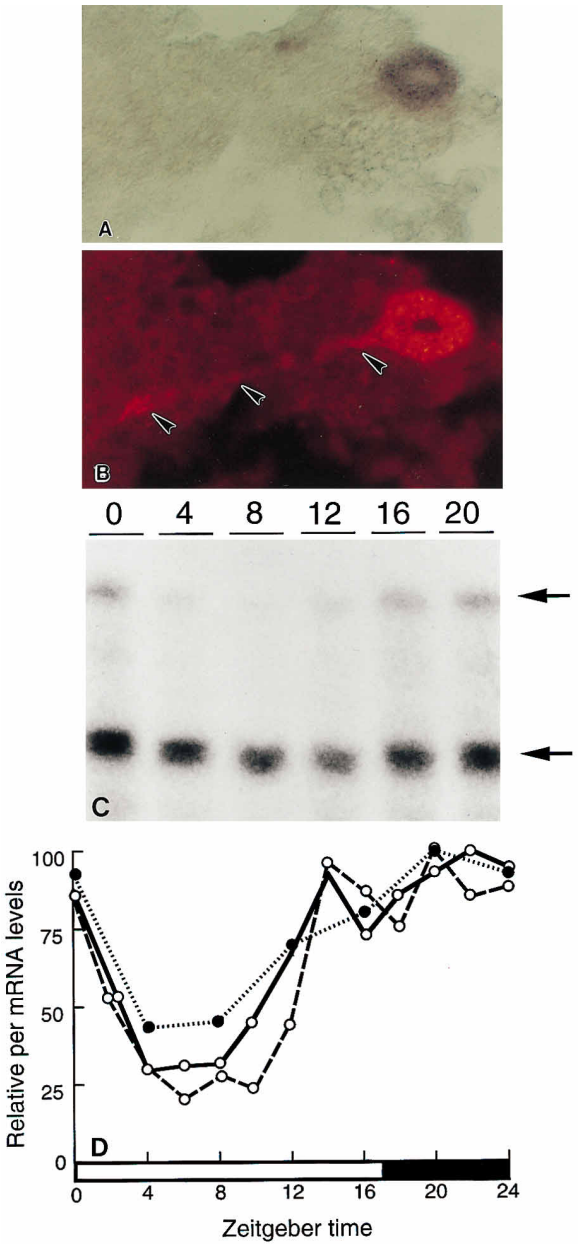

Figure 5. Colocalization of *per* mRNA with PER and Cycling of *per* mRNA Levels in A. pernyi Brain

(A and B) Simultaneous immunocytochemistry and in situ hybridization showing colocalization of *per* mRNA (blue, A) and PER (red, B) at ZT 12. Magnification, 405×.

(C) RNase protection assay of *per* mRNA levels examined throughout the lighting cycle. ZTs are depicted above each lane. Upper arrow, *per* protected fragment; lower arrow, RP49.

(D) Quantitation of RNase protection data for three separate experiments. Relative RNA levels refers to ratios of *per*:RP49 mRNAs that were converted to percentage of maximal level for each experiment. The horizontal bar represents the LD 17:7 lighting cycle.

was found for Per-c2A, whereas no hybridization signal was detected for Per-c2B (data not shown). Thus, the antisense transcript appears to be derived from the portion of the gene that encodes the amino half of PER.

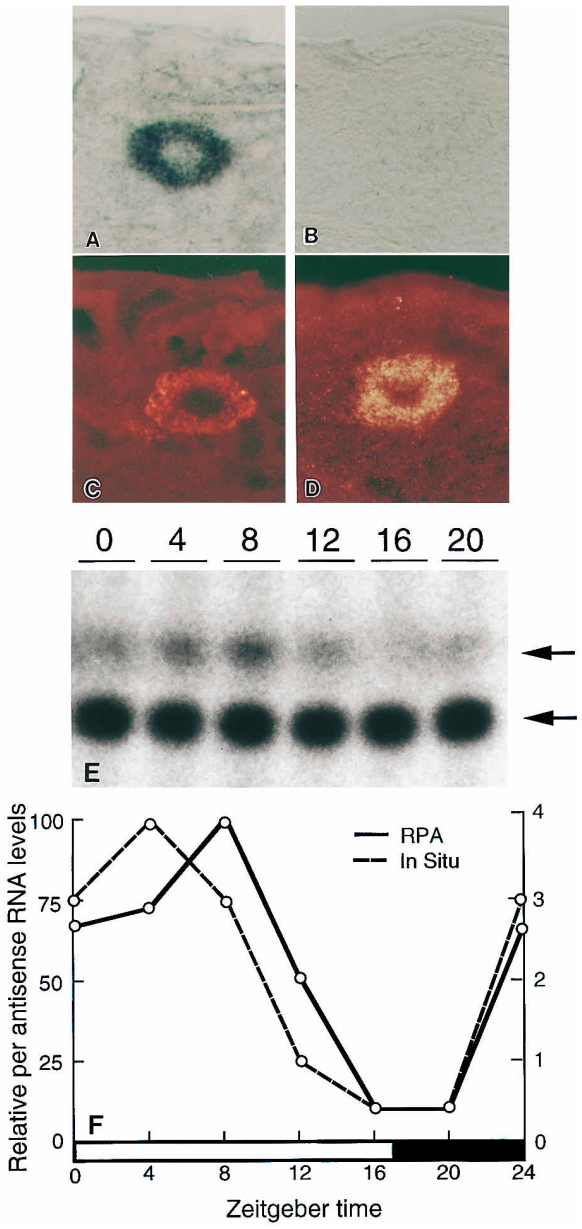

Figure 6. Colocalization of *per* Antisense RNA with PER and Cycling of Antisense RNA in A. pernyi Brain

(A–D) Simultaneous immunocytochemistry and in situ hybridization showing colocalization of PER (C and D) and antisense RNA (A and B) at ZT 8 (compare C and A) and at ZT 16 (compare D and B). Magnification, 420×.

(E) RNase protection assay of *per* antisense RNA levels examined throughout the lighting cycle. ZTs are depicted above each lane. Upper arrow, *per* antisense RNA; lower arrow, RP49.

(F) Quantitation of RNase protection and in situ hybridization data for one experiment. For RNase protection assays, relative RNA levels refer to ratios of *per*:RP49 mRNAs that were converted to percentage of maximal level for each experiment. For semiquantitation assessment of in situ hybridization, each value is the mean of three animals. No value varied from the mean by more than one level of intensity at each time. A similar hybridization pattern was observed in two other experiments. The horizontal bar represents the LD 17:7 lighting cycle.

PER and TIM Immunoreactivity Are Colocalized in Silkmoth Brain

In Drosophila, *tim* encodes a PER dimerization partner; TIM and PER are colocalized within photoreceptor and brain lateral neurons (Hunter-Ensor et al., 1996; Myers et al., 1996). Thus, if PER-positive cells in silkmoth brain are circadian clock cells, they would also be expected to express TIM. Since a silkmoth *tim* cDNA has not yet been cloned, we used two antibodies recently developed against Drosophila TIM (Hunter-Ensor et al., 1996; Myers et al., 1996) to examine TIM-like immunoreactivity in silkmoth brain. Both antibodies stained four pairs of cells in silkmoth brain (data not shown); TIM staining was more intense with one of the anti-TIM antibodies (Myers et al., 1996), so this antibody was used in all subsequent experiments. The location of the eight TIM-immunoreactive cells suggested that they might be the same ones that express PER. Indeed, double-labeling experiments showed that PER and TIM immunoreactivity are colocalized in eight neurons in silkmoth brain (Figures 7A and 7B).

As found for PER, TIM-immunoreactive cells showed a daily rhythm in the intensity of cytoplasmic staining (Figure 7C), and no staining was found in the nucleus at any of the timepoints examined. The temporal pattern of the oscillation in TIM immunoreactivity was virtually identical to that for PER (compare Figures 4D and 7C). Throughout LD 17:7, TIM staining was intense in axons. Axonal staining for TIM could be frequently traced all the way to the ipsilateral corpora allata (data not shown). Although the axonal staining for TIM was as intense as it was for PER (at the peak time), the daily variation in axonal staining was not nearly as striking for TIM as it was for PER.

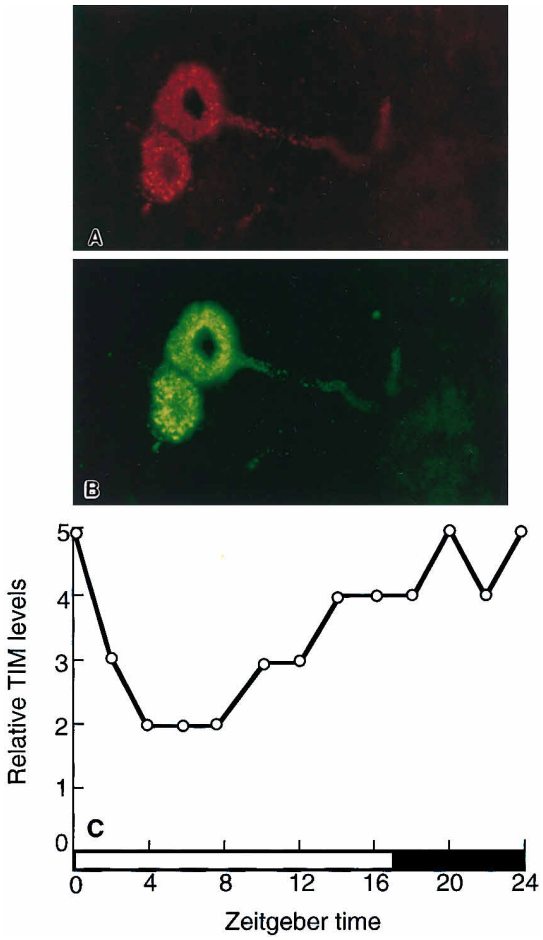

Figure 7. Colocalization of PER and TIM and Cycling of TIM Levels in A. pernyi Brain

(A and B) Double-labeling immunofluorescence of TIM (A) and PER (B). Magnification, 410×.

(C) Semiquantitative assessment of TIM immunostaining in brain cells (cytoplasm and axons) throughout LD 17:7. Each value is the mean of three animals. No values varied from the mean by more than one level of intensity at each time. A similar pattern was observed in two other experiments. The horizontal bar represents the LD 17:7 lighting cycle.

Clock Protein and mRNA Oscillations Are Suppressed by Constant Light

If PER-expressing cells in silkmoth brain are circadian clock cells involved in eclosion behavior, then these cells should be affected by constant light (LL), a treatment that abolishes the A. pernyi eclosion gate by the second day in LL (Truman, 1971). Indeed, LL abolished the adult eclosion gate and disrupted rhythms in PER, *per* sense mRNA, *per* antisense RNA, and TIM immunoreactivity (Figure 8). For each measure, LL exposure reduced levels to low to undetectable, with little fluctuation. The low, nonfluctuating levels for each measure strongly suggest that the individual rhythms were indeed dampened to arrhythmicity by LL. It is important to note that each of the measured rhythms was also monitored (at the times of high and low values in LD) for 1 or 2 days in constant darkness (DD). All rhythms persisted in DD with amplitudes similar to those observed in LD, showing that each rhythm is endogenously generated.

In contrast with the suppressive effects of LL on clock protein and mRNA oscillations, PTTH protein and mRNA levels in neurosecretory cells located within a few microns of the most lateral PER-expressing cells were not suppressed by LL (data not shown). Thus, the suppressive effect of LL does not extend to all neural systems in brain.

Relationship of PER-Expressing Cells to Neuropeptide-Expressing Cells in Silkmoth Brain

We also examined the relationship of PER-expressing cells to three peptidergic systems in silkmoth brain that are intimately associated with circadian function. PTTH was investigated because it is necessary for initiating adult development and its release is under circadian control (Williams, 1969). EH was examined because its release initiates adult eclosion behavior and is thus under circadian control (Truman, 1992). Pigment-dispersing hormone (PDH), a peptide isolated from crustaceans (Rao, 1992), was studied because it has been shown to colocalize with a subset of PER-expressing lateral neurons, which have been proposed to be circadian pacemaker cells in Drosophila brain (Helfrich-Forster, 1995).

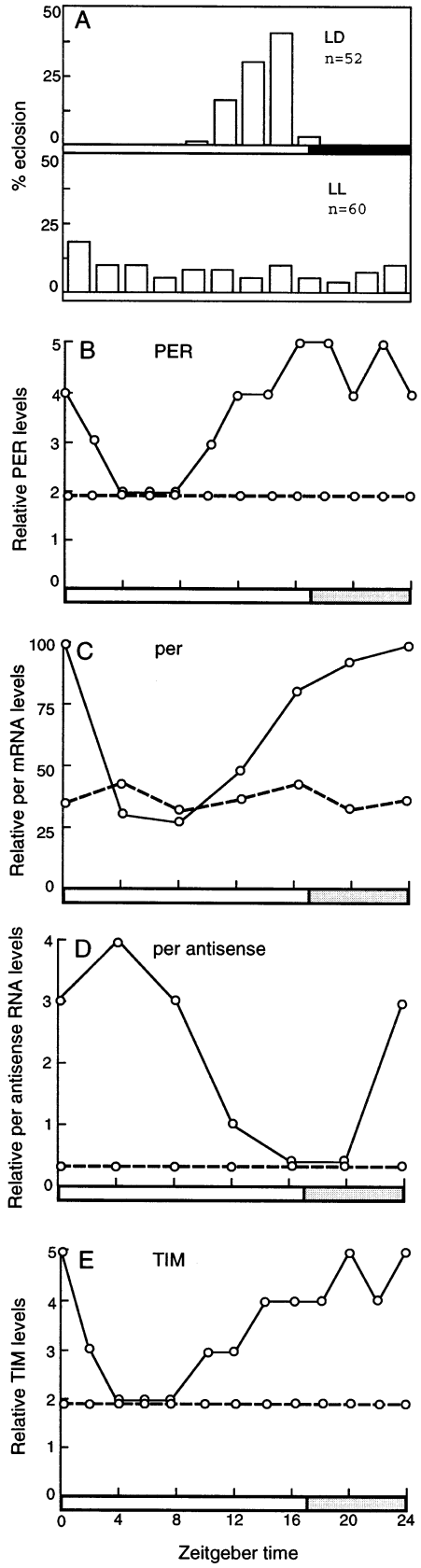

Figure 8. Effects of Constant Light on the Eclosion Gate and Clock Gene Oscillations in A. pernyi Brain

Pupae were maintained in LD 17:7 or placed into constant light (LL) 7 days before adult eclosion.

(A) Temporal profiles of adult eclosion in LD (upper) or LL (lower).

(B–E) Effects of LL on PER immunoreactivity (B), *per* mRNA (C), *per* antisense RNA (D), and TIM immunoreactivity (E) in neurons of dorsolateral protocerebrum. Dashed lines indicate values in LL. For reference, values from animals housed in LD are replotted (solid lines) from Figures 4, 6, and 7. LD and LL data for each measure were processed together. Each LL value is the mean of three animals. No values varied from the mean by more than one level of intensity at each time.

PTTH immunoreactivity occurred in a pair of cells adjacent to the lateral pair of PER-expressing cells (Figure 9A). Previous double-label studies have shown that PTTH and PER are not coexpressed in the same cells (Sauman and Reppert, 1996). PTTH-expressing cells send their axons to the contralateral corpora allata. A pair of EH-immunoreactive cells was found in the dorsal medial region of each brain hemisphere, with each pair sending ipsilateral projections through the subesophageal ganglion to the ventral nervous cord (Figure 9B). Axonal projections of PER-expressing cells and their arrborization were in the immediate vicinity of EH cell bodies. Small clusters of PDH-immunoreactive cells were found throughout silkmoth brain (Figure 9C). None of the PDH-positive cells were located near the eight PER-expressing cells in silkmoth brain.

Discussion

The results clearly show two distinct systems of PER regulation in the giant silkmoth, A. pernyi. In the eye, *per* mRNA and protein levels are expressed rhythmically, with a 4–6 hr temporal delay between the two rhythms. The oscillation of PER is due to its temporal appearance in the nuclei of photoreceptor cells. The temporal delay between the *per* mRNA and protein rhythms and the nuclear movement of PER in silkmoth eye are remarkably similar to the patterns observed in ocular photoreceptors and brain cells in Drosophila. Thus, the described PER regulatory system in silkmoth eye is quite consistent with the autoregulatory feedback loop hypothesis of PER developed in Drosophila (reviewed by Hall, 1995; Kay and Millar, 1995; Reppert and Sauman, 1995). It is not known, however, whether *per* oscillations are autonomous to silkmoth eye or whether these oscillations are driven by a brain clock. From previous studies, it is clear that the clock controlling circadian rhythms in adult eclosion and locomotor activity in silkmoths resides in brain, not in the eye (Truman and Riddiford, 1970; Truman, 1972, 1974).

The dynamics of PER regulation in silkmoth brain are strikingly different from PER regulation in the eye. PER is heavily expressed in the cytoplasm of eight brain cells, with no evidence of temporal movement into the nucleus (examined at 2 hr intervals throughout LD 17:7). The lack of PER staining in the nucleus is not due to problems with nuclear detection of PER, because the antibodies we used clearly detect PER in photoreceptor nuclei in the same brain sections. The pattern and cellular location of PER expression in brain is not peculiar to A.

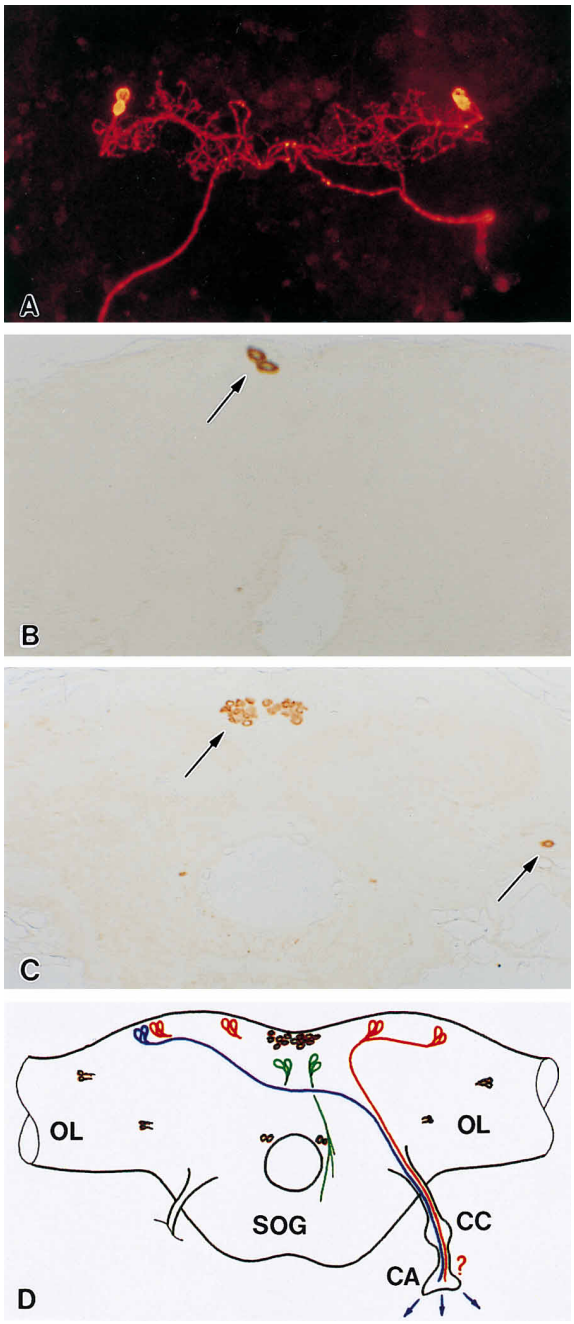

Figure 9. Relationship of PER-Expressing Cells to Three Neuropeptide-Containing Cells in A. pernyi Brain

(A) Wholemount immunofluorescence of PTTH-immunoreactive cells and their axonal projections. Magnification, 70×.
(B) EH-immunoreactive cells (arrows). Magnification, 85×.
(C) PDH-immunoreactive cells (arrows). Magnification, 50×.
(D) Schematic diagram illustrating the topography of PTTH-producing (blue), EH-producing (green), PDH-producing (black), and PER-producing (red) cells and their axonal projections in adult brain-retrocerebral complex; OL, optic lobe; SOG, suboesophageal ganglion; CA, corpus allatum; CC, corpus cardiacum.

pernyi, because we find an identical pattern of cytoplasmic PER expression (without nuclear staining) in several other saturniid moths (e.g., H. cecropia, A. luna, and A. polyphemus). Notably, the *per* mRNA and protein rhythms in A. pernyi brain appear synchronous, without an obvious temporal delay. The absence of a temporal delay between the *per* mRNA and protein levels argues against regulated nuclear movement of PER being important in decreasing *per* transcription in circadian clock cells in silkmoth brain. Synchronous protein and mRNA rhythms suggest that the mRNA rhythm directly drives the protein rhythm with only a small translational delay.

Several findings lead us to conclude that the PER-expressing cells in silkmoth brain are indeed circadian clock cells. These eight neurosecretory cells are the only cells in silkmoth brain that express PER, and the PER-positive cells are located in the dorsal lateral protocerebrum, the site of the circadian clock that drives rhythms in adult eclosion behavior and adult flight (Truman and Riddiford, 1970; Truman, 1972, 1974). In addition, these cells coexpress PER and *per* mRNA, indicating that they are indeed PER-expressing cells. Both PER and *per* mRNA also exhibit prominent circadian rhythms, which are canonical properties of a true clock element (Aronson et al., 1994; Hall, 1995). Furthermore, PER-expressing cells coexpress TIM-like immunoreactivity, suggesting that PER and TIM are colocalized and therefore interacting in silkmoth brain and that TIM is a second clock element for the silkmoth circadian system. Importantly, the measured molecular oscillations in the PER-positive cells in brain are all suppressed by LL, a treatment that disrupts the circadian gate of adult eclosion in A. pernyi (Truman, 1992) as well as circadian rhythms in Drosophila (Konopka et al., 1989). A final line of evidence in favor of the clock function of PER-positive brain cells is our recent finding that selective suppression of PER levels in A. pernyi embryos abolishes the circadian rhythm of egg hatching behavior (Sauman et al., 1996 [this issue of *Neuron*]).

There are substantial differences between PER-expressing cells in the brains of silkmoths and flies. In addition to the lack of nuclear movement of PER (and TIM) and the lack of a temporal delay between *per* mRNA and protein rhythms in silkmoth brain, the number of brain cells expressing PER is dramatically different between silkmoths and flies. In Drosophila, there are dozens of neurons and hundreds of glia that express PER (Ewer et al., 1992; Frisch et al., 1994), while there are only eight PER-expressing neurosecretory cells in silkmoth brain. The results of transgenic studies (Frisch et al., 1994) and genetic mosaic analysis (Ewer et al., 1992) suggest that a group of lateral neurons in Drosophila brain contain the relevant pacemaker cells for driving adult eclosion and locomotor rhythms. These PER-containing lateral neurons also stain for PDH, which has been proposed to be an output modulator of Drosophila circadian rhythms (Helfrich-Forster, 1995). In silkmoth brain, however, PER-expressing cells do not stain for PDH. PER-positive cells and their projections in silkmoth brain are nonetheless strategically positioned for regulating PTTH and EH release (Figure 9D). One of the most striking differences in PER staining between silkmoth and fly brain is the expression of PER in axons of silkmoth cells. Axonal and dendritic PER staining have been examined in Drosophila brain (Ewer et al., 1992), but not detected.

A novel function of PER in silkmoths suggested by its location in axons is that PER acts as a secreted factor to regulate circadian rhythms. The axonal pattern of PER staining shows that the four PER-positive cells in each brain hemisphere form a neural network, with axons from all four neurons coalescing to form one tract that projects to the ipsilateral corpora allata. We do not yet know, however, whether PER is found in secretory vesicles in axon terminals. PER is a large protein, but proteins as large as PER are secreted from Drosophila (e.g., Rothberg and Artavanis-Tsakonas, 1992). Alternatively, PER itself may not be secreted, but its presence in axonal terminals may affect the secretion of neuropeptides or transmitters that, in turn, affect circadian function. A similar scenario may also apply to axonal staining of TIM.

Without evidence of an autoregulatory transcription loop of PER regulation in silkmoth brain, how are *per* mRNA and protein rhythms generated? A unique mechanism suggested by our data in the silkmoth involves circadian regulation of PER by an antisense *per* transcript. Antisense *per* RNA was detected in A. pernyi brain by both in situ hybridization and RNase protection analysis using the same sense cRNA probe. The distribution of the *per* antisense transcript is spatially restricted to PER-expressing cells, suggesting that its functional role is limited to regulating PER.

Although antisense transcripts were first described in prokaryotes and viruses (reviewed by Inouye, 1988), their existence has been reported in a growing number of eukaryotic genes (Murashov and Wolgemuth, 1996). Even though the function of eukaryotic antisense RNAs has not been firmly established, prokaryotic and eukaryotic in vitro studies show that antisense RNA can regulate DNA replication, transcription, and translation (Kimelman, 1992). In some eukaryotic cells, antisense and sense mRNAs form RNA–RNA duplexes that could regulate RNA splicing or stability, block translation, interfere with mRNA transport to the cytoplasm, or covalently modify the sense mRNA. RNA–RNA duplexes seem a likely mechanism of *per* antisense function, because we have only detected the antisense transcript in the cytoplasm.

It is also possible that the *per* antisense transcript encodes a protein. However, examination of the noncoding strand of the *per* cDNA did not reveal the presence of any large open reading frames. Genomic analysis shows the existence of at least five introns in the amino half of the A. pernyi *per* coding region (data not shown), any of which could contain a promoter and all or a portion of an open reading frame on the complementary strand. There is no evidence of a tandem duplication of the *per* gene that could contain an inversion, because Southern blot analysis of A. pernyi genomic DNA suggests that the silkmoth *per* is a single copy gene (data not shown). Defining the molecular nature of the antisense RNA awaits isolation of its cDNA.

We propose that in the silkmoth the antisense transcript rhythm causes the decrease in sense mRNA levels and subsequent protein levels. Thus, circadian control of *per* may be regulated in part from circadian control of the antisense transcript. It is important to note that this mechanism may be more generalizable because we have found the same *per* antisense oscillation by RNase protection assay in silkmoth eye (data not shown). Therefore, the possible existence of an antisense *per* transcript should also be explored in Drosophila. It is noteworthy that an antisense transcript of unknown function has also been described for the Neurospora clock gene *frequency* (*frq*) (Dunlap et al., 1995).

We do not yet know what accounts for the different PER regulatory systems in A. pernyi eye and brain. It is possible that there are tissue-specific events that give rise to these two distinct systems. These tissue-specific mechanisms could be posttranscriptional differences in *per* and/or *tim* gene products, leading to modified proteins that affect nuclear transport or cytoplasmic localization. The recent development of an in vitro system for assessing PER–TIM interactions and mapping structural domains of both proteins involved in nuclear transport and cytoplasmic localization should prove useful for future assessment of the silkmoth proteins (Saez and Young, 1996 [this issue of *Neuron*]).

Autoregulatory transcriptional loops, first proposed for *per* in Drosophila, have been suggested to be a molecular theme fundamental to circadian clocks across diverse organisms. For example, in addition to *per* and *tim* molecular loops in Drosophila, the product of the Neurospora clock gene *frq*, known to encode a circadian clock element, also negatively regulates the level of its own transcript (Aronson et al., 1994). Since *per*, *tim*, and *frq* do not share significant sequence homology, it has been proposed that the basic mechanism of autoregulatory transcriptions loops is the same among diverse species, even though the specific molecular components vary. However, our findings of PER regulation in silkmoth brain suggest that autoregulatory feedback loops are not a universal mechanism of circadian clocks across metazoans. In addition, it now seems entirely possible that the same molecular component of a circadian clock may have diverse modes of regulation in different species.

Experimental Procedures

Animals

Diapausing pupae of A. pernyi were purchased from Worldwide Butterflies Limited (Sherborne, England) under United States Department of Agriculture permit number 929010. Domestic saturniid cocoons (H. cecropia, A. luna, and A. polyphemus) were purchased from Daniel Bantz (Caledonia, WI). Cocoons were stored in darkness at 4°C until use. To terminate diapause and initiate adult development, pupae were removed from cocoons and placed in environmental compartments at 24°C with the daily lighting cycle consisting of LD 17:7 (Williams and Adkisson, 1964). Adult development was completed within 3–4 weeks, followed by adult eclosion.

RNase Protection Assay

Adult central brain and eyes with optic lobes were separately dissected from CO_2-anesthetized animals, immediately placed on dry ice, and stored at −80°C until analysis. Total RNA was extracted from batches of eyes and brains (three for each tissue at each timepoint) using an Ultraspec RNA Isolation System (Biotex Labs). [^{32}P]UTP-labeled *per* cRNA antisense and sense probes (see Figure 2) were generated by subcloning PCR-amplified *per* cDNA fragments into pBluescript, followed by in vitro transcription driven from Sp6 and T7 RNA polymerase promoters, respectively. An antisense RP49 probe (153 bp) cloned from A. pernyi (Reppert et al., 1994) was included in each RNase protection reaction as a control for the amount of RNA loaded in each lane. To equalize the signal intensity of the *per* and RP49 bands on the gel, the specific activity of the

RP49 probe was decreased by diluting the [^{32}P]UTP 1:1000 with nonradioactive UTP in the in vitro transcription reaction.

RNase protection assays were performed using a kit from Ambion (RPAII) as previously described (Reppert et al., 1994). Quantitation was performed by directly counting radioactivity in the gel using a phosphorimager (Molecular Dynamics) and Image Quantitation (Molecular Dynamics) software (courtesy of S. Brown and R. Kingston, Massachusetts General Hospital). Each protection assay was performed twice with similar results. The results were replicated with at least two sets of animals for each experiment.

Antibody Production and Affinity Purification

Polyclonal antiserum was generated against a synthetic peptide corresponding to a fragment of the predicted amino acid sequence of A. pernyi PER (residues 605–618; KSSTETPLSYNQLN) corresponding to the "peptide S" region of D. melanogaster PER (Siwicki et al., 1988). The peptide was synthesized as a multiple antigen peptide (MAP) linked to a polylysine core. The MAP peptide was injected with Freund's incomplete adjuvant into two rabbits (Research Genetics). The resulting antiserum was subjected to immunoaffinity chromatography with the original synthetic peptide immobilized on the SulfoLink affinity column (Pierce). The specificity of the affinity-purified antibodies (57/10w and 58/10w) was tested by immunocytochemistry (see Results).

Immunocytochemistry

Silkmoth brains were dissected from CO_2-anesthetized animals and immediately fixed in modified Bouin–Hollande solution (Levine et al., 1995) overnight at 4°C. Standard histological techniques were employed for tissue dehydration, embedding in paraplast, sectioning (4–7 μm), deparaffinization, and rehydration. To remove residual heavy metal ions from the fixed tissue, the sections were treated with Lugol's iodine followed by 5% sodium thiosulfate. After thorough washing with distilled water and PBS supplemented with 0.2% Tween 20 and 0.1% bovine serum albumin (PBS-TB), the sections were blocked with 10% normal goat serum in PBS-TB (30 min at room temperature) and incubated with the desired primary antibody (appropriately diluted in PBS-TB) in a humidified chamber overnight at 4°C. Following rinsing with PBS-TB (three times for 10 min at room temperature), samples were incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch; 1:1000 in PBS-TB, 1 hr at room temperature). The HRP enzymatic activity was stained with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine·4HCl (0.25 mM in 0.05 M Tris–HCl [pH 7.5]) as chromogen. Stained sections were dehydrated and mounted in AccuMount-60 mounting medium.

The list of antibodies used for immunocytochemistry included the following: rabbit anti–A. pernyi PER (57/10w at 1:500); rabbit anti–A. pernyi PER (58/10w at 1:500); rabbit anti-"alfa"PER (D. melanogaster Baculovirus recombinant protein from M. Young, Rockefeller University; dilution 1:1,000); rabbit anti-PER (D. melanogaster E. coli recombinant protein from J. Hall, Brandeis University; dilution 1:1,000); rabbit anti-PER S80 (D. melanogaster synthetic peptide from K. Siwicki [Siwicki et al., 1988], Swarthmore College; dilution 1:300); rat anti-PAS (D. melanogaster E. coli recombinant protein from M. Rosbash, Brandeis University; dilution 1:150); rat anti-TIM #307 (D. melanogaster E. coli recombinant protein from M. Young [Myers et al., 1996], Rockefeller University; dilution 1:1,000); rat anti-TIM (D. melanogaster E. coli recombinant protein from A. Sehgal [Hunter-Ensor et al., 1996], University of Pennsylvania; dilution 1:500); rabbit anti–A. pernyi PTTH (274/IV-A [Sauman and Reppert, 1996]; dilution 1:4,000); rabbit anti-Manduca EH (from J. Truman, University of Seattle; dilution 1:200); and rabbit anti-Uca PDH (from H. Dircksen; dilution 1:10,000).

For double-labeling experiments, the primary antibodies (rabbit anti-PER [Young] 1:1000 in PBS-TB and rat anti-TIM [Young] 1:11,000 in PBS-TB) as well as the corresponding secondary antibodies (goat anti-rabbit IgG-Cy3 conjugated and goat anti-rat IgG-Cy2 conjugated) were combined and applied to the brain sections under the same conditions as described above for single antibody labeling. The stained sections were mounted and viewed under a Nikon microscope equipped with epifluorescence and Nomarski optics.

In control experiments, the primary antibodies were replaced with normal goat serum. As an additional control for binding specificity, anti-PER antibodies (57/10w and 58/10w) were preincubated with 100 molar excess of antigen prior to immunological staining. In all cases, no significant staining was observed above background.

For scoring of immunoreactive intensities, stained sections were coded and viewed under a microscope. Levels of staining were subjectively scored with an intensity scale from 0 to 5. The time of collection was decoded after scoring. All the rhythms assessed by immunocytochemistry with diaminobenzidine were confirmed using immunofluorescence.

Brain Wholemount Immunofluorescence

Adult brains of A. pernyi were dissected as described above for immunocytochemistry and fixed immediately in aqueous Bouin fixative for 2–4 hr at 4°C. Samples were rinsed briefly in PBS, and the neurilemma was manually removed under a dissecting microscope. Brains were then permeabilized in PBS containing 2% Tween 20 (PBS-Tw) with several changes overnight at 4°C. Following blocking with 20% normal goat serum in PBS-Tw (2 hr at room temperature), brains were incubated with the primary anti-PTTH antibody (1:1000 in PBS-Tw) for 24–48 hr at 4°C. After thorough washing with PBS-Tw (three times for 20 min at room temperature), samples were incubated with Cy3 fluorophore conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch; 1:500 in PBS-Tw, 2 hr at room temperature), rinsed thoroughly in PBS-Tw (three times for 30 min at room temperature), mounted in 75% glycerol, and viewed under a Nikon microscope equipped with Nomarski optics and epifluorescence.

In Situ Hybridization

Digoxigenin-labeled antisense and sense probes (Per-c2 fragment; Figure 2) were generated by subcloning a PCR-amplified fragment of the silkmoth *per* cDNA into pBluescript followed by in vitro transcription in the presence of digoxigenin–UTP driven from Sp6 and T7 RNA polymerase promoters, respectively. The efficiency of digoxigenin incorporation and sensitivity of the labeled probes were assessed by detection on nylon membranes.

Dissected brains and developing embryos of A. pernyi were fixed in freshly made paraformaldehyde solution (5% in 0.1 M sodium phosphate buffer [pH 7.5]) for 2–6 hr at room temperature or overnight at 4°C. Samples were washed thoroughly with the same buffer and processed for sectioning as described above for immunocytochemistry, but omitting the Lugol's iodine and sodium thiosulfate treatments. Rehydrated sections on Vectabond (Vector Labs) coated slides were treated with 0.2 N HCl (20 min at room temperature), acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated, and hybridized with digoxigenin-labeled RNA probes in a hybridization cocktail (final concentration 0.5 ng/μl) overnight at 55°C. The hybridization solution consisted of 50% deionized formamide, 10% dextran sulfate, 2× Denhart's solution, 50 mM sodium phosphate buffer (pH 7.0), 2× SSC, 0.1% SDS, 5 mM EDTA, 0.1% sodium pyrophosphate, 500 μg/ml sonicated salmon sperm DNA, and 500 μg/ml yeast tRNA.

Following hybridization, the sections were washed with 2× SSC supplemented with 0.05% sodium pyrophosphate and 1 mM EDTA (SSC-NE; two times for 30 min at room temperature), digested with RNase A (10 μg/ml in 10 mM Tris–HCl [pH 8.0], 0.5 M NaCl), and rinsed again with 2× SSC-NE (30 min at room temperature). The final washes were done with 0.1× SSC-NE (two times for 30 min at 55°C, and two times for 20 min at room temperature). The immunocytochemical detection of hybridized probes with Fab fragments of sheep anti-digoxigenin antibody directly conjugated to AP (Boehringer Mannheim; 1:500 dilution in PBS-TB) was performed under virtually the same conditions as described above for immunocytochemistry. The AP activity was detected with the BCIP/NBT substrate system. Dehydrated slides were mounted in AccuMount-60 mounting medium.

For double-labeling experiments with anti-PER antibody, the hybridized brain sections, following the AP staining, were washed thoroughly in PBS-TB, blocked with 10% normal goat serum in PBS-TB (30 min at room temperature), and incubated with primary anti-PER antibody (1:500 in PBS-TB) overnight at 4°C. Slides were then

washed with PBS-TB (three times for 10 min), and the binding of the primary antibody was detected with goat anti-rabbit IgG-Cy3 conjugated secondary antibody (1:500 in PBS-TB, 1 hr at room temperature).

For scoring of hybridization intensities, stained sections were coded and viewed under a microscope. Levels of staining were subjectively scored with an intensity scale from 0 to 4. The time of collection was decoded after scoring.

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